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Whole-Cell Detection of C−P Bonds in Bacteria

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Supporting Information

ABSTRACT: Naturally produced molecules possessing a C−P bond, such as phosphonates and phosphinates, remain vastly underexplored. Although success stories like fosfomycin have reinvigorated small molecule phosphonate discovery efforts, bioinformatic analyses predict an enormous unexplored biological reservoir of C−P bond-containing molecules, including those attached to complex macromolecules. However, high polarity, a lack of chromophores, and complex macromolecular association impede phosphonate discovery and characterization. Here we detect widespread transcriptional activation of phosphonate biosynthetic machinery across diverse bacterial phyla and describe the use of solid-state nuclear magnetic resonance to detect C−P bonds in whole cells of representative Gram-negative and Gram-positive bacterial species. These results suggest that phosphonate tailoring is more prevalent than previously recognized and set the stage for elucidating the fascinating chemistry and biology of these modifications.

Biologically synthesized molecules possessing reduced phosphorus are relatively rare and poorly understood. For example, biological phosphonates, which possess a C−P bond but otherwise resemble phosphate, were not discovered until 1959.[1] Indeed, despite successful natural products like fosfomycin and phosphinothricin, relatively few of these compounds have been characterized.[2−4] Furthermore, rapidly accumulating genomic data increasingly suggest that molecules harboring C−P bonds are more biologically ubiquitous and chemically diverse than previously recognized.[5,6]

The biological ubiquity and diversity of phosphonates have been predicted from phylogenetic relationships among phosphoenolpyruvate (PEP) mutase enzymes encoded by *ppm* genes.[5,6] PEP mutase (Ppm) catalyzes C−P bond formation by rearranging PEP to phosphonopyruvate (PnPy) as the common first biosynthetic step en route to most phosphonates and phosphinates (Figure 1A).[7,8] Phylogenetic analysis revealed a widespread genetic capacity for phosphonate biosynthesis among microbes and predicted the existence of a large reservoir of undiscovered phosphonates, approximately half of which may be bioactive small molecules. The remaining ∼50% of the identified *ppm*-containing microbial gene clusters were predicted to synthesize phosphonolipids or phosphonoglycans associated with the cell wall.[5] Intriguingly, although several such molecules have been isolated from a variety of organisms, their biological roles remain enigmatic.[4]

Our limited understanding of phosphonate biology can be partly attributed to the chemistry of phosphonates. For instance, a high polarity and poor ultraviolet−visible absorption often prevent detection by traditional natural product workflows favoring chromophore-rich hydrophobic molecules, a bias reflected in the plethora of isolated polyketide, nonribosomal peptide, and terpene natural products.[9] Alternative approaches can detect phosphonates in polar aqueous fractions but require significant sample processing[10,11] and do not capture insoluble macromolecular phosphonates like phosphonolipids and phosphonoglycans. For example, extracellular polysaccharides, biofilms, and cell wall materials are notoriously insoluble and intractable to compositional analysis.[12] Furthermore, although bioinformatics approaches have detected widespread genetic capacity for phosphonate biosynthesis,[5] new methods are needed to easily identify the corresponding phosphonate products.

Solid-state nuclear magnetic resonance (NMR) is a powerful platform for quantitatively defining cell-wall and extracellular matrix composition.[13−15] In contrast to the extensive sample preparation (e.g., enzymatic digestion) required for traditional high-performance liquid chromatography−mass spectrometry workflows, solid-state NMR can be employed to analyze complex and insoluble systems, such as whole cells and biofilms, to more accurately reflect in vivo chemistry.[16] Solid-state NMR is particularly well suited for detecting C−P bonds in whole cells because of their characteristically downfield ^{31}P chemical shift.[17] Herein, we use reverse transcription polymerase chain reaction (RT-PCR) and solid-state NMR to reveal active phosphonate biosynthetic clusters in both Gram-positive and Gram-negative bacteria.

RESULTS AND DISCUSSION

Two anaerobes of the human microbiome were selected for analysis because they (i) contain phosphonate biosynthetic genes, (ii) are distantly related (Figure S1A), and (iii) inhabit the subgingival crevice of periodontitis patients and therefore affect human health. *Olsenella uli* is a Gram-positive actinobacterium prevalent in periodontitis, and its post-treatment persistence in the root canal suggests a role in chronic

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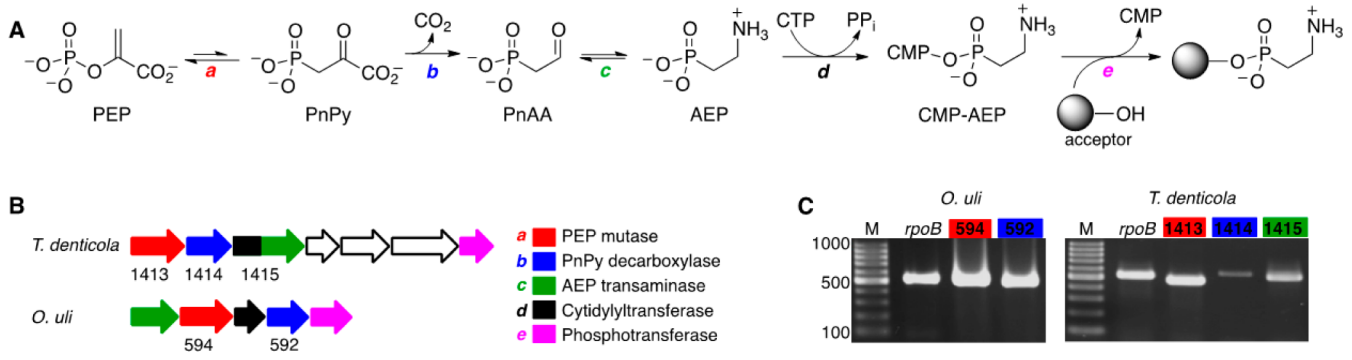

Figure 1. Proposed chemical logic of phosphonate biosynthesis based on bioinformatic and gene expression analyses. (A) Proposed pathway for AEP biosynthesis (PnAA, phosphonoacetaldehyde; AEP, 2-aminoethylphosphonate; CMP-AEP, cytidylylmonophosphate-AEP). (B) Phosphonate biosynthetic gene clusters for *Treponema denticola* and *Olsenella uli*, with functional annotations of enzymes *a*–*e* summarized in panel A. (C) Agarose gels of DNA amplicons from RT-PCR (M, molecular weight marker; *rpoB*, positive control). The remaining amplicons are color-coded and numbered as in panel B. Molecular marker sizes are in base pairs.

endodontic infections.[18−20] *Treponema denticola* is a Gram-negative spirochete that is highly enriched in periodontitis.[21] Respective phosphonate biosynthetic pathways (Figure 1A) can be proposed on the basis of features common to both gene clusters (Figure 1B). Specifically, we predict 2-aminoethylphosphonate (AEP) as the biosynthetic product of genes encoding PEP mutase (*ppm*), PnPy decarboxylase (*ppd*), and AEP transaminase (*aept*).[4] The presence of cytidylyltransferases suggests cytidylylmonophosphate (CMP) activation of AEP prior to phosphotransferase-catalyzed installation on a larger scaffold. Significantly, these cytidylyltransferases are homologous to LicC from *Streptococcus pneumoniae*, which uses CTP to generate CDP-choline from choline phosphate,[22] and are also predicted to activate *O*-methyl phosphoramidate for capsular polysaccharide modification in *Campylobacter jejuni*.[23]

To initially evaluate phosphonate biosynthetic capacity under the growth conditions tested, we monitored gene transcription by RT-PCR. Amplicons of 400−600 bp were detected with high primer specificity for all transcripts (Figure 1C). The gene encoding a DNA-dependent RNA polymerase β subunit (*rpoB*) was used as a positive control to confirm robust detection of a constitutively expressed gene.[24] Although the *ppm* gene was clearly expressed in both organisms, the *ppd* band intensity in *T. denticola* was significantly diminished relative to that of the *ppm* amplicon. We therefore sought additional evidence of phosphonate biosynthetic gene cluster activation by screening for transcription of the gene encoding transaminase−cytidylyltransferase fusion protein TDE1415 (Figure 1C). The greater yield of this *aept* amplicon relative to that of the *ppd* amplicon confirmed phosphonate biosynthesis gene transcription in *T. denticola* under the growth conditions that were tested.

Inspired by consistent transcriptional activation in *T. denticola* and *O. uli*, we explored the extent of transcriptional activity of phosphonate biosynthetic machinery in additional species. Specifically, we cultured the anaerobic actinobacterium *Atopobium rimae*, the anaerobic firmicute *Oribacterium sinus*, and the aerobic proteobacteria *Burkholderia thailandensis* and *Burkholderia vietnamiensis*. Surprisingly, phosphonate biosynthetic genes were activated in all species under the standard growth conditions (Figure S2), implying potentially constitutive C−P bond installation among diverse members of the bacterial tree of life (Figure S1A).

We next sought to identify possible macromolecular scaffolds targeted for phosphonylation in each strain. Because Ppm sequence similarity more closely correlates with gene cluster similarity (and therefore predicted product) than species of origin, we evaluated Ppm sequence phylogeny as previously described by Yu et al.[5] (Figure S1B). The Ppm of *T. denticola* clusters with putative group 3 phosphonoglycans, while the Ppm sequences of *A. rimae*, *O. uli*, and *Or. sinus* cluster adjacent to group 4 putative phosphonolipids. Interestingly, *B. thailandensis* and *B. vietnamiensis* Ppm sequences belong to group 1 of the putative phosphonolipids and clearly possess gene clusters predicted to encode production of 1-hydroxy-2-aminoethylphosphonate-modified lipids (Figure S1C).

Encouraged by phosphonate gene cluster transcriptional activation, we sought to obtain direct evidence of the production of C−P bond-containing molecules in our two prioritized strains of interest, *T. denticola* and *O. uli*. Although such molecules might be detected through extensive cellular fractionations and analysis by solution-based methods, our predicted C−P bonds could be included in insoluble parts of the cell and not detected. In addition, extraction yields of accessible C−P bond-containing molecules could be low. Thus, we examined whole-cell samples by ^{31}P cross-polarization magic-angle spinning (CPMAS) solid-state NMR. In addition to *T. denticola* and *O. uli*, we cultured *Escherichia coli* for analysis as a negative control because it lacks known phosphonate biosynthesis genes. Each cell sample was frozen and lyophilized for analysis. The 202 MHz ^{31}P NMR spectrum for *E. coli* illustrates the typical ^{31}P NMR pattern observed for cellular phosphates with multiple spinning sidebands surrounding the ^{31}P centerband and sideband spacing corresponding to the magic-angle spinning speed of 7143 Hz (Figure 2). The ^{31}P spectra for *O. uli* and *T. denticola* have an additional phosphorus contribution at 21.1 ppm corresponding to a carbon-bonded phosphorus (Figure 2). A ^{31}P CPMAS NMR spectrum was also acquired for *O. uli* at 6250 Hz to confirm assignment of the centerbands, which do not change frequency with altered spinning speed (Figure 2, inset, dashed blue). The prevalence of the *O. uli* phosphonate contributions relative to the phosphate signal was also observed to be higher in the *O. uli* whole-cell sample than in the *T. denticola* sample, consistent with their respective RT-PCR band intensities for *ppd*. However, phosphates come from various sources in a cell. These include major contributions from lipids and rRNA. The

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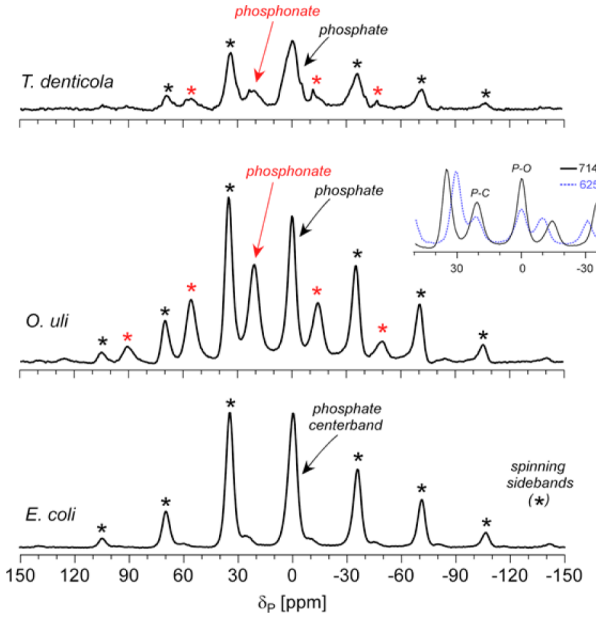

Figure 2. ^{31}P CPMAS solid-state NMR analysis of lyophilized whole cells. Phosphonate and phosphate centerbands are annotated, and sidebands are identified by asterisks. ^{31}P NMR was performed at 202 MHz, and spectra were the result of approximately 130000 scans. Magic-angle spinning was performed at 7143 Hz except where indicated in the inset spectrum.

exact concentrations of ribosomes and lipids are not known precisely for these samples. The exact lipid composition and contributions from lipopolysaccharides can also differ among species. Thus, we do not utilize the ratio of phosphate to phosphonate for a quantitative determination of phosphonate concentration but rather as an indicator of the presence or absence of phosphonates. In summary, solid-state NMR enabled the direct detection of C−P bonds in *O. uli* and *T. denticola*.

CONCLUSIONS

Since the discovery of naturally occurring phosphonates almost 60 years ago, they have achieved success as a minor but very biologically active class of small molecule natural products.[2−4] However, recent bioinformatic analyses have revealed a mosaic of natural C−P bonds broader than that previously recognized, with perhaps the majority existing not as small molecules but instead as modifications of large cell surface glycans and lipids.[5] Bacterial cell walls and extracellular matrices play an important role in persistence and pathogenicity because they present the initial contact surface for nutrients, signaling molecules, antibiotics, host tissues, and immune systems. Moreover, the tailoring of bacterial cell walls with various small molecules is increasingly recognized as a critical mechanism of virulence and host immune evasion.[25,26] Of particular note with respect to the present study, phosphoethanolamine and choline phosphate have been extensively implicated in virulence,[27−29] and the structurally related homologue AEP may play similar roles in various organisms.

Thus, we have revealed the transcriptional activation of phosphonate biosynthetic machinery in diverse bacteria and demonstrated the utility of coupling bioinformatic and transcriptional analyses with solid-state NMR to identify the physical presence of C−P bonds in whole cells. This approach allows rapid and sensitive screening of complex and insoluble cellular materials, avoids perturbative extraction protocols for initial detection, and sets the stage for deciphering the fascinating chemistry and biology of phosphonate tailoring in microbial cell walls.

ASSOCIATED CONTENT

Supporting Information

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Detailed experimental procedures, lists of primers used, and bioinformatic analyses (PDF)

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Notes

The authors declare no competing financial interest.

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